

CURRENT TOPIC

Nitric oxide and infectious diseases

David Burgner, Kirk Rockett, Dominic Kwiatkowski

Nitric oxide (NO) has undergone something of an image change in recent years. Previously considered a pollutant from car exhausts, NO has now been implicated in many physiological processes. Here we review the dual roles of NO in infection—as a critical agent of host defence but also as a central mediator of pathogenesis—using septic shock, bacterial meningitis, and malaria to illustrate some current concepts and controversies. We have limited our discussion of the important physiological functions of NO in the cardiovascular and neurological systems to instances where these impinge on its role in infectious diseases.

NO is formed by the oxidative deamination of the amino acid L-arginine by nitric oxide synthases (NOS) (fig 1). Three isoforms of this enzyme are described (table 1).¹ Neuronal NOS (nNOS or NOS1) is constitutively present in both the central and peripheral nervous systems, where NO acts as a neurotransmitter. Endothelial NOS (eNOS or NOS3) is constitutively expressed by endothelium and other cell types and is involved in cardiovascular homeostasis. In contrast, inducible NOS (iNOS or NOS2) is absent in resting cells, but the gene is rapidly expressed in response to stimuli such as proinflammatory cytokines. Once present, iNOS synthesises 100–1000 times more NO than the constitutive enzymes and does so for prolonged periods; the production of NO by eNOS and nNOS has been likened to a dripping tap, while that by

iNOS to a fire hose. This high concentration of NO may inhibit a large variety of microbes, but may also potentially damage the host, thereby contributing to pathology.

Antimicrobial effects of NO

NO inhibits the growth of many bacteria and parasites *in vitro*.² The antimicrobial effect stems not from NO itself, but from reactive nitrogen intermediates formed by the oxidation of NO. For example, reaction between NO and the free radical superoxide (O_2^-) results in the formation of the unstable molecule peroxynitrite ($OONO^-$), while that between NO and thiol groups produces nitrosothiols. These reactive nitrogen intermediates inactivate key microbial enzymes, such as ribonucleotide reductase and aconitase, by reacting with iron containing groups in these enzymes. Studies have shown that murine macrophages can produce sufficient NO to kill leishmania parasites *in vitro*.³ *In vivo* experiments using inhibitors of iNOS, and more recently knockouts of the iNOS gene, have clearly shown its role in the control of murine infections as diverse as malaria,⁴ leishmaniasis,⁵ tuberculosis,⁶ and listeriosis.⁷

How much iNOS is produced by human leucocytes?

Although these observations undoubtedly indicate the potential importance of high concentration NO as an antimicrobial agent, a fundamental question remains as to the cellular

Molecular Infectious Diseases Group,
Department of Paediatrics, University of Oxford, Level 4,
John Radcliffe Hospital, Oxford OX3 9D, UK
D Burgner
K Rockett
D Kwiatkowski

Correspondence to:
Dr Burgner.
email: dburgner@molbiol.ox.ac.uk

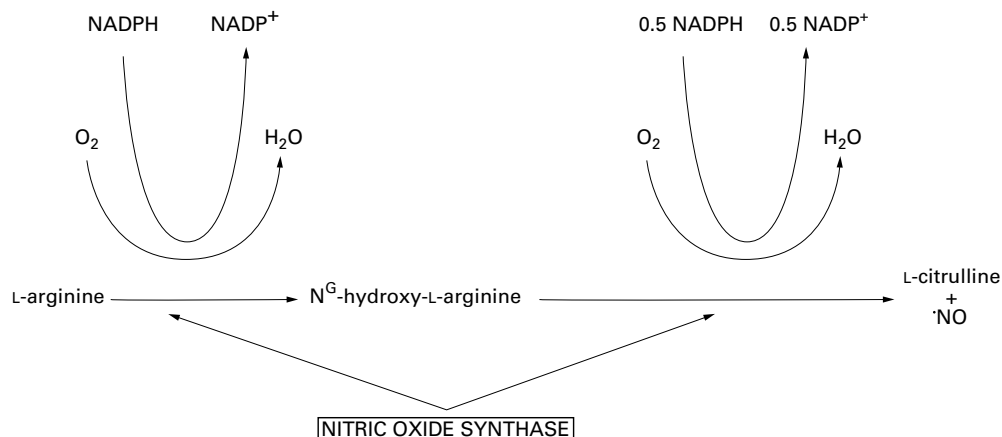


Figure 1 Conversion of L-arginine to L-citrulline plus nitric oxide as catalysed by nitric oxide synthases. Two primary steps have been identified. The first step, a two electron oxidation, is a hydroxylation of one of the guanidino nitrogens of L-arginine requiring molecular oxygen and nicotinamide adenine diphosphate (NADPH) to form N^G-hydroxy-L-arginine. The second step is a three electron oxidation, again requiring molecular oxygen and NADPH to perform an electron removal, oxygen insertion, and carbon-nitrogen bond scission to form L-citrulline and the free radical nitric oxide.

Table 1 Some characteristics of human nitric oxide synthases (NOS) (enzyme classification EC 1.14.23)

<i>Isoform</i>	<i>Alternative names</i>	<i>Molecular weight (kD) as monomer*</i>	<i>Intracellular location</i>	<i>Human cellular sources</i>	<i>Presence</i>	<i>Activation</i>
nNOS	Neuronal NOS ncNOS NOS1 NOS type I	155	Membrane associated	Neurons, adrenal medullary, renal macula densa, glia, astrocytes,	Constitutive	Ca ⁺⁺ increase leading to calmodulin binding
iNOS	Inducible NOS NOS2 NOS type II	125–135	Cytosolic	Macrophages, monocytes, leukocytes, endothelium, smooth muscle, neutrophils, retinal pigmented epithelium, astrocytes, microglial, hepatocytes, Küpffer cells, fibroblasts, mesangium	Inducible	Transcriptional induction Ca ⁺⁺ independent, calmodulin always bound
eNOS	Endothelial NOS ecNOS NOS3 NOS type III	135	Membrane (inactive) Cytosolic (active)	Endothelium, platelets, smooth muscle	Constitutive	Ca ⁺⁺ increase leading to calmodulin binding

*All enzymes active as dimers.

source of iNOS derived NO in people. In mice it is well established that NO is produced by macrophages, but in humans this evidence has proved more elusive. The human iNOS gene can be expressed in various cell types (table 1), but it has been much more difficult to demonstrate high levels of iNOS expression by leucocytes,⁸ leading some to question the relevance of NO in human infection.⁹ However, there is a growing view that this may reflect the greater complexity of iNOS regulation, and possibly a greater degree of stimulus and tissue specificity in humans compared to mice.¹⁰ Whereas it is extremely difficult to stimulate leucocytes from healthy humans to produce iNOS in vitro, leucocytes from patients with inflammatory or infectious diseases have been shown both to express iNOS and to produce large amounts of NO. For example, NO producing leucocytes have been found in the peripheral blood of patients with septic shock¹¹ and malaria,¹² and in bronchoalveolar lavage fluid from patients with tuberculosis.¹³ Thus the issue is not whether human leucocytes can make NO, but what stimuli are required and whether they do so in sufficient quantities to have a significant antimicrobial and pathological impact.

Investigating NO production in disease

Most studies rely on reactive nitrogen intermediates (in practice measurement of nitrite and nitrate (NO_x) in plasma or urine) as a surrogate for NO production. However NO_x concentrations are profoundly affected by diet, renal function, and hydration, all of which may be altered in severe illness.¹² This has made interpretation of studies difficult and yielded conflicting results, especially when patients selected as controls have conditions which also result in iNOS expression. In addition, systemic concentrations of NO_x may not reflect local production of NO in key organs, such as the spleen, bone marrow, or central nervous system. Measurements of other intermediates (such as nitrosothiols and nitrotyrosine, which reflect NO production and are not influenced by dietary nitrate), immunohistochemical investigation of iNOS expression, or investigation of genetic differences in the ability to produce

NO may prove more useful in investigating the extent and site of NO production in inflammatory states.

NO and septic shock

Under physiological conditions eNOS derived NO activates soluble guanylate cyclase in vascular smooth muscle, increasing intracellular cyclic guanosine monophosphate and causing vasodilation. In sepsis, iNOS derived NO may mimic and exaggerate this process, causing chronic vasodilation, as well as interfering with and damaging other pathways. This makes iNOS an attractive candidate as a mediator of shock and multiorgan failure in sepsis. Animal models confirm widespread iNOS expression and NO production in response to Gram negative bacteria, endotoxin or inflammatory cytokines,¹⁴ resulting in hypotension, organ failure, and death. These effects are prevented by non-selective NOS inhibitors, such as N^G-monomethyl-L-arginine (L-NMMA), which reverses systemic hypotension and improves survival in a number of animal models.¹⁵ iNOS knockout mice, in which the iNOS gene has been experimentally disrupted, are protected from endotoxin induced hypotension in some studies,⁷ although they are more susceptible to overwhelming infection with a number of intracellular organisms.¹⁰

There is good evidence for iNOS induction and increased NO production in human septic shock.¹¹ Initial reports of the use of non-selective NOS inhibitors (such as L-NMMA) in those with refractory sepsis induced hypotension showed an increase in mean arterial pressure and systemic and pulmonary vascular resistance.¹⁶ Enthusiasm waned when it emerged that non-selective NOS inhibition actually increased mortality in septic patients, possibly by its negative effects on cardiac output and organ perfusion.¹⁷ This discrepancy between animal and human studies highlights the need to extrapolate animal studies cautiously. Intraperitoneal or intravenous infusion of a known amount of endotoxin or bacteria obviously differs from human infection, where the inoculum and stage of infection are unknown. Many animal studies pretreat with NOS inhibitors, before bacterial challenge,

whereas in human trials pathological processes may be well advanced at presentation. More fundamentally, inhibition of a biological system with so many important physiological functions might be expected to have widespread and deleterious consequences. Current interest has focused on selective iNOS inhibitors, which might ameliorate the pathological effects of inappropriate NO production, but with less disruption of normal physiology. Although animal data support this approach,¹⁸ a large trial of a selective iNOS inhibitor (546C88)¹⁷ has produced disappointing results.

NO and meningitis

In bacterial meningitis, meningeal inflammation is initiated by local production of proinflammatory cytokines, especially tumour necrosis factor (TNF) and interleukin 1 β ,¹⁹ which are both potent stimuli of iNOS expression in leucocytes, glia, and neurones. Cerebrospinal fluid NO_x concentrations are increased in experimental²⁰ and human meningitis, correlating with cerebrospinal fluid TNF concentrations in the latter.²¹ Immunohistochemical staining of postmortem tissue from experimental animals²⁰ and humans (IA Clark, personal communication, 1999) show widespread iNOS expression and perimeningeal NO production. As before, NO may have a protective and pathological role; iNOS knockout mice are more susceptible to experimental *Listeria monocytogenes* infection,⁷ and inhibition of NO worsens outcome in some models of bacterial meningitis, leading to focal cerebral ischaemia.²⁰ Conversely many of the pathological changes seen in bacterial meningitis, such as disruption of the blood-brain barrier, cerebral oedema, changes in cerebral blood flow,²² and damage to cochlear cells,²³ can be prevented experimentally by NOS inhibitors. Dexamethasone reduces iNOS transcription in vitro and this might contribute to its protective effect in reducing cochlear damage and thus residual deafness. Whether NO acts as a neurotoxic or neuroprotective molecule may be dependent on its redox state; in its oxidised form (NO⁺) inactivates glutamate receptors and reduces the neurotoxicity of excitatory amino acids, whereas the reduced form (NO \bullet) can form peroxynitrate, a potent neurotoxin.²⁴ The therapeutic use of NOS inhibitors in bacterial meningitis has been suggested, but as NO is likely to have profound effects on cerebral blood flow and other important physiological processes, our understanding of the role of NO in meningitis seems inadequate to justify this intervention at present.

NO and malaria

As in many parasitic infections, NO appears central to the host response in malaria, mediating the antiparasitic effects of proinflammatory cytokines at each stage of the parasite life cycle in both experimental and possibly human infection.²⁵ In addition to its protective role, NO has been suggested as a mediator in cerebral malaria. Cerebral malaria is the most infamous of the severe manifestations of *Plasmodium falciparum* infection, causing

around 1 million deaths per year. The pathogenesis of cerebral malaria is debated, but derives from the sequestration of parasitised erythrocytes in the cerebral vasculature, where they may cause microvascular obstruction, local induction of inflammatory cytokines, or both. Clinically, there is often rapid recovery from profound coma without significant neurological sequelae, implying that a reversible process contributes to coma. It has been suggested that NO, produced by iNOS induced in vascular endothelium by proinflammatory cytokines, may cross the blood-brain barrier and affect local neuronal function, by mimicking and exaggerating the physiological effects of endogenous nNOS derived NO.²⁵ Investigating this hypothesis has proved difficult, as plasma concentrations of NO_x do not reflect local NO production within the brain. Some studies find that high concentrations of plasma NO_x correlate with depth and length of coma in cerebral malaria,²⁶ while others suggest that high plasma concentrations are protective against severe disease.¹² CSF NO_x, which may be more reflective of local cerebral production and is less affected by diet and other confounders, is increased in cerebral malaria.²⁷ Preliminary immunohistological studies suggest widespread iNOS induction in cerebral endothelium and resultant NO production (IA Clark, personal communication, 1999), although suitable control samples for such studies are difficult to obtain. Different genetic polymorphisms of the iNOS promoter region have been associated with increased risk of death from cerebral malaria in Gambian children²⁸ and with protection from severe malaria in Gabonese children,²⁹ suggesting that complex genetic factors may determine iNOS production and thereby influence clinical outcome.

Conclusions

The L-arginine NO pathway is a vital antimicrobial defence mechanism. Because of the ubiquitous nature of this pathway in normal physiology, it is easy to see how high and continuous NO production in sepsis might contribute to pathology, either by interfering with NO dependent pathways or by causing direct tissue injury. Our knowledge of the role of NO in sepsis is incomplete. The regulation of iNOS expression is complex and may be cell type, differentiation stage, and stimulus dependent, making the extrapolation of animal studies to the clinical setting difficult. As our understanding increases, modulation of the NO L-arginine pathway in infection may become a valuable therapeutic option.

- 1 Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. *Cell* 1994;78:915-8.
- 2 De Groote MA, Fang FC. NO inhibitions: antimicrobial properties of nitric oxide. *Clin Infect Dis* 1995;21(suppl 2):S162-5.
- 3 Liew FY, Millott S, Parkinson C, Palmer RM, Moncada S. Macrophage killing of leishmania parasite in vivo is mediated by nitric oxide from L-arginine. *J Immunol* 1990;144:4794-7.
- 4 Seguin MC, Klotz FW, Schneider I, et al. Induction of nitric oxide synthase protects against malaria in mice exposed to irradiated *Plasmodium berghei* infected mosquitoes: involvement of interferon gamma and CD8⁺ T cells. *J Exp Med* 1994;180:353-8.

- 5 Wei XQ, Charles IG, Smith A, *et al.* Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 1995;375:408–11.
- 6 MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci USA* 1997;94:5243–8.
- 7 MacMicking JD, Nathan C, Hom G, *et al.* Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 1995;81:641–50.
- 8 Denis M. Human monocytes/macrophages: NO or no NO? *J Leukoc Biol* 1994;55:682–4.
- 9 Schoedon G, Schneemann M, Walter R, Blau N, Hofer S, Schaffner A. Nitric oxide and infection: another view. *Clin Infect Dis* 1995;21(suppl 2):S152–7.
- 10 Nathan C. Inducible nitric oxide synthase: what difference does it make? *J Clin Invest* 1997;100:2417–23.
- 11 Tsukahara Y, Morisaki T, Horita Y, Torisu M, Tanaka M. Expression of inducible nitric oxide synthase in circulating neutrophils of the systemic inflammatory response syndrome and septic patients. *World J Surg* 1998;22:771–7.
- 12 Anstey NM, Weinberg JB, Hassanali MY, *et al.* Nitric oxide in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. *J Exp Med* 1996;184:557–67.
- 13 Nicholson S, Bonecini-Almeida MdG, Lapa e Silva JR, *et al.* Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J Exp Med* 1996;183:2293–302.
- 14 Wong ML, Rettori V, al-Shekhlee A, *et al.* Inducible nitric oxide synthase gene expression in the brain during systemic inflammation. *Nat Med* 1996;2:581–4.
- 15 Hollenberg SM, Cunliffe RE, Zimmerberg J. Nitric oxide synthase inhibition reverses arteriolar hyporesponsiveness to catecholamines in septic rats. *Am J Physiol* 1993;264:H660–3.
- 16 Petros A, Bennett D, Vallance P. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet* 1991;338:1557–8.
- 17 Ketteler M, Cetto C, Kirdorf M, Jeschke GS, Schafer JH, Distler A. Nitric oxide in sepsis syndrome: potential treatment of septic shock by nitric oxide synthase antagonists. *Kidney Int Suppl* 1998;64:S27–30.
- 18 Aranow JS, Zhuang J, Wang H, Larkin V, Smith M, Fink MP. A selective inhibitor of inducible nitric oxide synthase prolongs survival in a rat model of bacterial peritonitis: comparison with two nonselective strategies. *Shock* 1996;5:116–21.
- 19 Ramilo O, Saez-Llorens X, Mertsola J, *et al.* Tumor necrosis factor alpha/cachectin and interleukin 1 beta initiate meningeal inflammation. *J Exp Med* 1990;172:497–507.
- 20 Leib SL, Kim YS, Black SM, Tureen JH, Tauber MG. Inducible nitric oxide synthase and the effect of aminoguanidine in experimental neonatal meningitis. *J Infect Dis* 1998;177:692–700.
- 21 van Furth AM, Seijmonsbergen EM, Groeneveld PH, van Furth R, Langermans JA. Levels of nitric oxide correlate with high levels of tumor necrosis factor alpha in cerebrospinal fluid samples from children with bacterial meningitis. *Clin Infect Dis* 1996;22:876–8.
- 22 Koedel U, Bernatowicz A, Paul R, Frei K, Fontana A, Pfister HW. Experimental pneumococcal meningitis: cerebrovascular alterations, brain edema, and meningeal inflammation are linked to the production of nitric oxide. *Ann Neurol* 1995;37:313–23.
- 23 Amaec FR, Comis SD, Osborne MP, Drew S, Tarlow MJ. Possible involvement of nitric oxide in the sensorineural hearing loss of bacterial meningitis. *Acta Otolaryngol (Stockh)* 1997;117:329–36.
- 24 Lipton SA, Choi YB, Sucher NJ, Chen HS. Neuroprotective versus neurodestructive effects of NO-related species. *Biofactors* 1998;8:33–40.
- 25 Clark IA, Rockett KA. Nitric oxide and parasitic disease. *Adv Parasitol* 1996;37:1–56.
- 26 Al-Yaman FM, Mokela D, Genton B, Rockett KA, Alpers MP, Clark IA. Association between serum levels of reactive nitrogen intermediates and coma in children with cerebral malaria in Papua New Guinea. *Trans R Soc Trop Med Hyg* 1996;90:270–3.
- 27 Weiss G, Thuma P, Biemba G, Mabeza G, Werner ER, Gondek VR. Cerebrospinal fluid levels of biotin, nitric oxide metabolites, and immune activation markers and the clinical course of human cerebral malaria. *J Infect Dis* 1998;177:1064–8.
- 28 Burgner D, Xu W, Rockett KA, Gravenor M, *et al.* Inducible nitric oxide synthase polymorphism and fatal cerebral malaria. *Lancet* 1998;352:1193–4.
- 29 Kun JF, Mordmuller B, Lell B, Lehman LG, Luckner D, Kremsner PG. Polymorphism in promoter region of inducible nitric oxide synthase gene and protection against malaria. *Lancet* 1998;351:265–6.